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(57) Abstract

The present invention provides a method for making R stereospecific alkyl- and aryl-phosphonate linkages between nucleotides. These methods can be used for automated synthesis of oligonucleotides having sequential R stereospecific alkyl- and arylphosphonate linkages. The present invention is also directed to the oligonucleotides having several sequential R phosphonate linkages which were produced by the subject methods. Moreover, the present invention provides methods for using the subject oligonucleotides, including methods for regulating the biosynthesis of a DNA, an RNA or a protein and methods for detecting and isolating complementary nucleic acid targets.

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TRIVALENT SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING STEREOSPECIFIC ALKYLPHOSPHONATES AND ARYLPHOSPHONATES

The present invention provides methods of making R-stereospecific alkyl- or aryl-phosphonate linkages between nucleotides. Moreover, these methods are amenable to automation. The present invention is also directed to the R-stereospecific alkyl- and aryl-phosphonate oligonucleotides formed by such methods. Moreover, in another embodiment, the present invention is directed to methods of using the R-stereospecific oligonucleotides, for example, as diagnostic probes and as therapeutic agents having the capability of regulating cellular and viral DNA replication, RNA transcription, protein translation, and other processes involving nucleic acid templates. Furthermore, the present R-stereospecific oligonucleotides can be used as probes for detection or isolation of a target nucleic acid.

Oligonucleotides have been employed diversely in utilities ranging from diagnosis and therapy of disease to discovery, cloning and synthesis of nucleic acids. For example, oligonucleotides can be used as probes to identify target nucleic acids that are present in vivo, in tissue samples or that are immobilized onto a filter or membrane. After identification by the oligonucleotide, a target nucleic acid can be cloned and an oligonucleotide can be used to prime the synthesis of that nucleic acid. Moreover, hybridization patterns of an oligonucleotide to a nucleic acid that differ from normal hybridization patterns are frequently useful in diagnosis of disease. Furthermore, there has been great

interest recently in developing oligonucleotides as therapeutic agents which can regulate the biological function of cellular or viral nucleic acids.

Interest in oligonucleotides as therapeutic agents has arisen from observations of naturally occurring complementary, or antisense, RNA used by some cells to control protein expression. More recently, synthetic oligonucleotides have been used with success to inhibit gene expression. For example, oligonucleotides were initially utilized to inhibit growth of Rous sarcoma virus (Zamecnik et al. 1978 Proc. Natl. Acad. Sci. USA 75: 280-284). Since such initial studies, oligonucleotides have been used to inhibit the expression of a wide variety of target nucleic acids in both cell-free extracts and in whole cells derived from diverse organisms, including viruses, bacteria, plants and animals. For example, expression of vesicular stomatitis virus matrix protein, human c-myc protooncogene, and c-Ha-ras protooncogene has been inhibited by oligonucleotides (Wickstrom et al. 1986 Biophys. J. 49: 15-19; Heikkila et al. 1987 Nature 328: 445-449; Wickstrom et al. 1988 Proc. Natl. Acad. Sci. USA 85: 1028-1032; and Daaka et al. 1990 Oncogene Res. 5: 267-275). A review of such therapeutic applications for oligonucleotides is provided by Uhlmann et al. 1990, Chemical Reviews 90: 543-584.

However, the development of oligonucleotides for in vivo regulation of biological processes has been hampered by several long-standing problems, including the nuclease sensitivity and poor cell penetrability of oligonucleotides.

In contrast to normal phosphodiester ($O-PO_2-O$) linkages present in common, naturally occurring nucleic acids, both R and S stereoisomeric aryl- or alkyl-substituted phosphonate linkages confer several desirable properties upon an oligonucleotide, including increased nuclease resistance and increased cell penetration. Moreover, oligonucleotides having racemic alkylphosphonate linkages have been shown to specifically inhibit growth of simian virus 40, vesicular stomatitis virus, herpes simplex virus type 1 and human immunodeficiency virus (Miller et al. 1985 Biochimie 67: 769-776; Agris et al. 1986 Biochemistry 25: 6268-6275; Smith et al. 1986 Proc. Natl. Acad. Sci. USA 83: 2787-2791; and Sarin et al. 1988 Proc. Natl. Acad. Sci. USA 85: 7448-7451).

However, relatively high concentrations of alkyl- or aryl-phosphonate oligonucleotides have been required to achieve a significant therapeutic effect. This requirement for high oligonucleotide concentrations is apparently due to inefficient binding by oligonucleotides which have some phosphonate linkages in the S-stereospecific configuration (Miller 1991 Biotechnology 9: 358-362). S-stereospecific linkages are generated in addition to R-stereospecific linkages using presently available non-stereospecific synthetic procedures.

In particular, replacement of one of the phosphate oxygens with another group, so that four different groups are attached to the phosphorous atom, generates a chiral phosphate which can exist in two stereo-configurations, R and S (Rp and Sp, respectively). Current synthetic procedures are non-

stereospecific and typically generate a linkage having either a Rp or Sp configuration, as each nucleotide is added, to thereby generate an oligonucleotide having a mixture of Rp and Sp linkages. However, the melting temperatures of pure Rp and Sp isomers differ significantly, with the Rp isomer binding much more strongly than the Sp isomer (Miller et al. 1980 J. Biol. Chem. 235: 9659-9665; and Lesnikowski et al. 1990 Nucleic Acids Res. 18: 2109-2115). Hence, oligonucleotides with Rp phosphonate linkages have highly desirable binding properties and consequently greater utility than oligonucleotides with Sp or racemic phosphonate linkages.

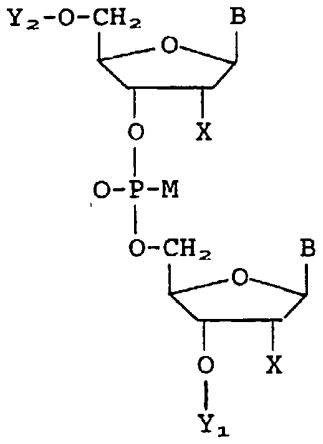
Moreover, a procedure which efficiently produces such highly desirable Rp isomer linkages on alkyl- or aryl-phosphonate oligonucleotides presents a large improvement over available prior art procedures.

Present methods for obtaining oligonucleotides with only Rp alkyl- or aryl-phosphonate linkages require steps that are not readily adapted to automation, are inefficient or can be used for obtaining very short oligonucleotides, i.e. oligonucleotides having only up to about 8 oligonucleotides. For example, Lesnikowski et al. (1988 Nucleic Acids Res. 16: 11675-11689) have reported stereospecific dimer, trimer and tetramer synthesis of oligonucleotides using Grignard reagent activation of the 5'-OH group nucleotide and purification of Rp and Sp isomers after addition of each nucleotide. However, these methods present formidable difficulties for automation. More recently, Lesnikowski et al. (1990 Nucleic Acids Res. 18: 2109-2115) have reported synthesis of an octamer

$(dT)_8$ with a central racemic methylphos-phonate linkage and with other linkages as either all Rp or all Sp. Lebedev et al. (1990b Tetrahedron Letters 31: 855-858) provide a method for making single stereospecific phosphonothioate (i.e. P-S-C-5') linkages between two nucleotides. However, to date there is no disclosure of a method which permits efficient automated synthesis of Rp-stereospecific alkyl- or aryl-phosphonate (i.e. P-O-C-5') linkages.

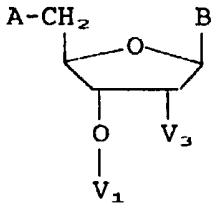
The present invention provides efficient methods for synthesis of Rp stereospecific alkyl- and aryl-phosphonate linkages between nucleotides of an oligonucleotide. Moreover, the present methods can readily be adapted for automated oligonucleotide synthesis. The present invention is also directed to Rp isomeric oligonucleotides produced by these methods, and to methods of using the present Rp alkyl- or aryl-phosphonate oligonucleotides as diagnostic probes and as therapeutic agents.

The present invention is directed to a method for producing an oligonucleotide having an Rp stereoisomeric alkyl- or aryl-phosphonate linkage between a first nucleotide and a second nucleotide in the oligonucleotide, wherein the oligonucleotide is of the formula:

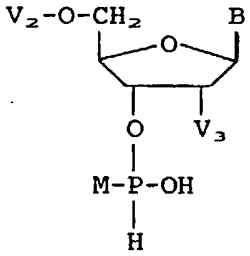


which comprises:

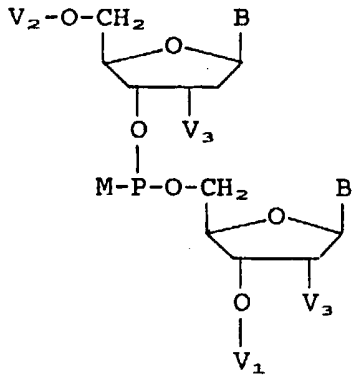
(a) reacting a 5'-O-activated nucleotide of the formula:



with an alkyl- or aryl-phosphinate nucleotide intermediate of the formula:



under conditions sufficient to produce an Sp stereoisomeric alkyl- or aryl-phosphonate linkage of the formula:



wherein:

Y_1 is a hydrogen, phosphate, phosphate present in the oligonucleotide or V_1;

Y_2 is a hydrogen, phosphate, phosphate present in the oligonucleotide or V_2;

X is hydroxy or V_3;

V_1 is a protecting group, a solid support or a phosphate attached to a penultimate nucleotide of the oligonucleotide;

V_2 is a protecting group;

V_3 is hydrogen or $O\text{-}Y_3$ wherein Y_3 is lower alkyl or protecting group;

M is a lower alkyl, cycloalkyl, thioxo, a thio-lower alkyl, aryl or aryl-lower alkyl group which can be substituted with at least one hydroxy, halogen or cyano group;

each B group is independently a purine or pyrimidine base which can have 1-3 substituents selected

from the group consisting of lower alkyl, amino, oxo, hydroxy, lower alkoxy, amino-lower alkyl, lower alkylamino, hydroxy-lower alkyl, aryl and aryl lower alkyl;

A is an activating group;

the intermediate has an Sp phosphorus stereoisomeric configuration; and

(b) reacting the Sp linkage with an oxidizing agent under conditions sufficient to produce Rp stereoisomeric alkyl- or aryl-phosphonate linkage; and

(c) when V_1, V_2 or V_3 is a protecting group, optionally removing said V_1, V_2 or V_3 protecting group.

The present invention also relates to a method of producing a polynucleotide chain of an oligonucleotide having at least one Rp alkyl-phosphonate or one Rp aryl-phosphonate linkage.

The present invention further relates to an alkyl- or aryl-phosphonothioate nucleotide intermediate, wherein the intermediate has an Sp stereoisomeric phosphorus configuration. Such an intermediate can be used to generate the present Rp stereoisomeric linkages.

The present invention still further relates to a compartmentalized kit for producing a polynucleotide chain of an oligonucleotide having at least five Rp alkyl-phosphonate or Rp aryl-phosphonate linkages.

The present invention also relates to an oligonucleotide having at least five Rp alkyl-phosphonate or Rp aryl-phosphonate linkages produced by the subject methods.

The present invention further relates to the present oligonucleotides which have an attached agent to facilitate cell delivery, a drug or a reporter molecule.

The present invention still further relates to a compartmentalized kit for detection or diagnosis of a target nucleic acid.

The present invention additionally relates to a compartmentalized kit for isolation of a template nucleic acid.

The present invention also relates to a method of regulating biosynthesis of a DNA, an RNA or a protein using the subject Rp alkyl- or aryl-phosphonate oligonucleotides.

The present invention further relates to a pharmaceutical composition for regulating biosynthesis of a nucleic acid or protein comprising a pharmaceutically effective amount of one of the present oligonucleotides and a pharmaceutically acceptable carrier.

The present invention still further relates to a method of detecting a target nucleic acid which includes contacting one of the present oligonucleotides with a sample to be tested for containing such a nucleic acid for a time and under conditions sufficient to form an oligonucleotide-target complex; and detecting such a complex.

Fig. 1 depicts a chromatograph of Rp and Sp stereoisomers of dithymidine methylphosphonate separated by liquid chromatography on a 4.6 x250 mm C_{18} silica column with gradient elution using 10% to 15% acetonitrile in water (0.25%/min) at a flow rate of 1.0 ml/min.

Fig. 2 depicts superimposed circular dichroism spectra of Rp and Sp dithymidine methylphosphonate stereoisomers separated as illustrated in Fig. 1. Each

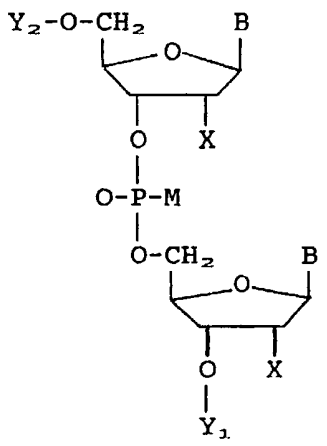
stereoisomer has a characteristic spectrum which can be used to identify that stereoisomer.

Fig. 3 depicts ^{1}H NMR spectra of Rp (top) and Sp (bottom) stereoisomers of dithymidine methylphosphonate, illustrating several distinct peaks characteristic of a given stereoisomer which can be used for stereoisomeric identification, e.g. the H_2 and H_6 peaks.

Fig. 4 depicts ^{31}P NMR spectra of Rp (top) and Sp (bottom) stereoisomers of dithymidine methylphosphonate. The Rp stereoisomer has a characteristic additional peak at 7.984 ppm which can be used to identify this stereoisomer.

Fig. 5 depicts a spectrograph of 5'-dimethoxytrityl-tetrathymidine methylphosphonate-3'-acetate (DMT-TpTpTpT-OAc) produced by fast atom bombardment mass spectroscopy (FABMS). Specific peaks corresponding to distinct molecular fragments of DMT-TpTpTpT-OAc are identified (e.g. 5'-dimethoxytrityl-dithymidine, DMT-TpT, at 850 m/e).

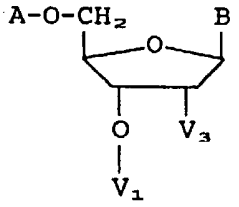
The present invention provides a method for producing an oligonucleotide having an Rp stereoisomeric alkyl- or aryl-phosphonate linkage between a first nucleotide and a second nucleotide in the oligonucleotide, wherein the oligonucleotide is of the formula:



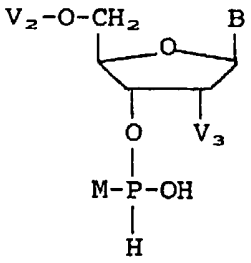
I

According to the present invention, Rp stereoisomeric alkyl- or aryl-phosphonate linkages between two nucleotides are formed by:

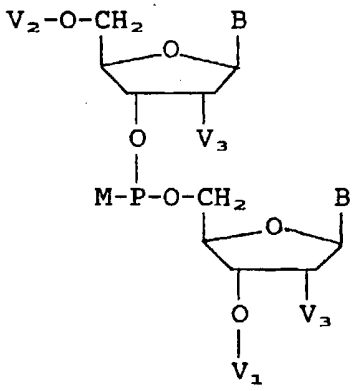
(a) reacting a 5'-O-activated nucleotide of the formula:



with an alkyl- or aryl-phosphinate nucleotide intermediate of the formula:



under conditions sufficient to produce an Sp stereoisomeric alkyl- or aryl-phosphonate linkage of the formula:



wherein:

Y_1 is a hydrogen, phosphate, phosphate present in the oligonucleotide or V_1;

Y_2 is a hydrogen, phosphate, phosphate present in the oligonucleotide or V_2;

X is hydroxy or V_3;

V_1 is a protecting group, a solid support or a phosphate attached to a penultimate nucleotide of the oligonucleotide;

V_2 is a protecting group;

V_3 is hydrogen or $O-Y_3$ wherein Y_3 is lower alkyl or protecting group;

M is a lower alkyl, cycloalkyl, thioxo, a thio-lower alkyl, aryl or aryl-lower alkyl group which can be substituted with at least one hydroxy, halogen or cyano group; and

each B group is independently a purine or pyrimidine base which can have 1-3 substituents selected

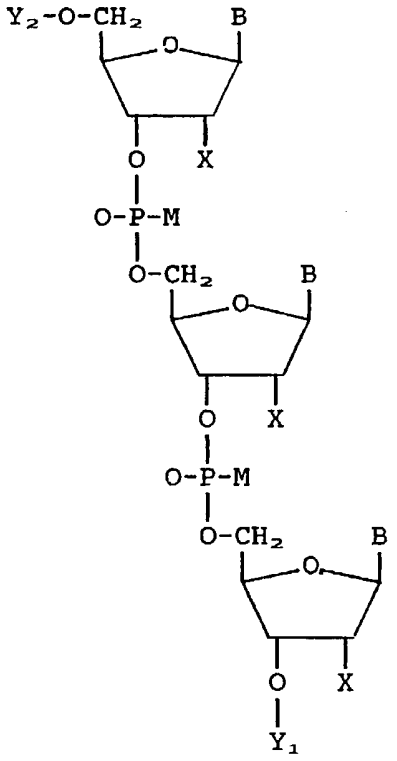
from the group consisting of lower alkyl, amino, oxo, hydroxy, lower alkoxy, amino-lower alkyl, lower alkylamino, hydroxy-lower alkyl, aryl and aryl lower alkyl; and

A is an activating group;

(b) reacting the Sp linkage with an oxidizing agent under conditions sufficient to produce the Rp stereoisomeric alkyl- or aryl-phosphonate linkage; and

(c) when V_1, V_2 or V_3 is a protecting group, optionally removing said V_1, V_2 or V_3 protecting group.

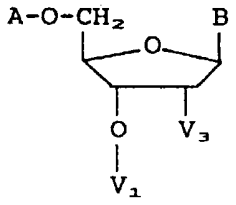
In another embodiment, the present invention provides a method of producing a polynucleotide chain of an oligonucleotide having at least one Rp-alkyl-phosphonate or Rp-aryl-phosphonate linkage, wherein the oligonucleotide has the formula:



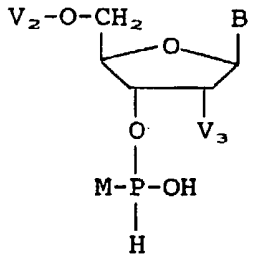
II

The present method for producing at least one Rp-alkyl-phosphonate or Rp-aryl-phosphonate linkage in a polynucleotide chain of an oligonucleotide includes the following steps:

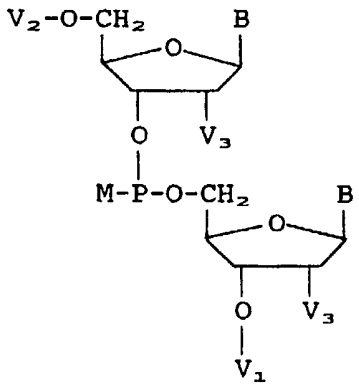
(a) reacting a 5'-O-activated nucleotide of the formula:



lwith an alkyl- or aryl-phosphinate nucleotide intermediate of the formula:



under conditions sufficient to produce an Sp stereoisomeric alkyl- or aryl-phosphonate linkage of the formula:



wherein:

Y_1, Y_2, X, V_1, V_2, V_3, M and B are as defined hereinabove; and

n is an integer of from 0 to 200;

the intermediate has an Sp phosphorus stereoisomeric configuration; and

A is an activating group present on the 5'-activated oxygen;

(b) reacting the Sp linkage with an oxidizing agent under conditions sufficient to produce the Rp stereoisomeric alkyl- or aryl-phosphonate linkage and so generate a new 5'-terminal nucleotide;

(c) removing the V_2 protecting group from the new 5'-terminal nucleotide;

(d) activating a 5'-oxygen on the new 5'-terminal nucleotide to generate a new 5' activated oxygen;

(e) reacting the product of (d) with another alkyl- or aryl-phosphinate nucleotide intermediate under conditions sufficient to produce another Rp stereoisomeric linkage and to generate a new 5'-terminal nucleotide;

(f) repeating steps c, d and e to extend the polynucleotide chain n-1 times; and

(g) when V_1, V_2 or V_3 is a protecting group, optionally removing said V_1, V_2 or V_3 protecting group.

If the desired product is a compound of Formula I or II wherein X is OH and Y_1 or Y_2 are hydrogen or phosphate, such groups are generated upon removal of the protecting groups by standard techniques known to one skilled in the art.

The Rp stereoisomeric alkyl- or aryl-phosphonate linkages produced by the methods of the present invention have M substituents on the phosphate atom. Such an M substituent is present instead of an oxygen atom commonly found in conventional nucleic acids which have $-O-PO_2-O-$ linkages. According to the present invention, M is a lower alkyl, a cycloalkyl, a thioxo, a thio-lower alkyl, an aryl or an aryl lower alkyl group wherein such lower alkyl and aryl groups can be

substituted with at least one hydroxy, halogen or cyano group.

As used herein the term lower alkyl refers to alkyl groups containing one to six carbon atoms. Lower alkyl groups can be straight-chained or branched, and include such moieties as methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, iso-butyl, t-butyl, pentyl, amyl, hexyl and the like. Preferred M alkyl groups of the present invention have from one to four carbon atoms. The most preferred M alkyl group is methyl. Similarly, a lower alkenyl is a lower alkyl with 1-3 carbon-carbon double bonds.

Moreover, an alkoxy group is a lower alkyl attached via an oxygen atom; a lower acyl is a lower alkyl attached via a carbonyl (C=O); and a lower cyanoalkyl is a lower alkyl with a CN substituent.

The term cycloalkyl refers to saturated cyclic structure, i.e. a ring, having 3-7 ring carbon atoms. Cycloalkyl groups contemplated by the present invention include cyclopropyl, cyclo-butyl, cyclopentyl, cyclohexyl, cycloheptyl rings and the like.

A thioxo group is a =S group and a thio-lower alkyl is a lower alkyl attached to the phosphate via a sulfur atom.

The term aryl refers to an aromatic moiety containing 6-10 ring carbon atoms and includes phenyl, α-naphthyl, ß-naphthyl, and the like. A preferred aryl group is phenyl.

An aryloxy group is an aryl attached via an oxygen atom and an aroyl is an aryl attached via a carbonyl (CO). Similarly, an aryloxyacyl is an aryl linked to an acyl via an oxygen atom.

According to the present invention a halo group is a halogen. Halo groups include fluorine, chlorine, bromine and iodine. A preferred halo group for substitution on M lower alkyl, aryl, and aryl lower alkyl groups is fluorine.

Preferred M groups are lower alkyl or phenyl groups which can be substituted with a halo group, preferably a fluorine. More preferred M groups are unsubstituted lower alkyl groups. An especially preferred M group is an unsubstituted methyl group. Therefore, the preferred Rp-stereoisomeric linkages of the present invention are alkylphosphonate linkages and more preferably are methylphosphonate linkages.

According to the present invention, the nucleotides joined by the present alkyl- or aryl-phosphonate linkages can have deoxyribose or ribose sugar moieties. Therefore, as defined herein X is either hydroxy or V_3, wherein V_3 is hydrogen, or O-Y_3 and Y_3 is lower alkyl or a protecting group. Accordingly, when X is hydrogen a deoxyribose sugar is present but when X is hydroxy or -O-Y_3 a ribose sugar, an O-alkyl ribose sugar or a protected ribose sugar, is present in the associated nucleotide. Preferred oligonucleotides of the present invention have X as hydrogen or hydroxy. However, during synthesis of the present oligonucleotides such a hydroxy is protected with a protecting group, which can be removed at conclusion of synthesis by the present methods.

The nucleotides linked according to the present invention each have a B group which represents the base moiety present on the nucleotide. Each B group is independently a purine or pyrimidine base which can

have 1-3 substituents independently selected from the group consisting of lower alkyl, amino, oxo, hydroxy, lower alkoxy, amino-lower alkyl, lower alkylamino, hydroxy-lower alkyl, aryl and aryl lower alkyl.

Preferred B groups of the present invention are purines such as guanine (G) and adenine (A), and pyrimidines such as thymine (T), cytosine (C) or uracil (U). In addition, preferred B groups include any related base analog that is capable of base pairing with a guanine, adenine, thymine, cytosine or uracil. For example, such base analogs include pseudocytosine, isopseudocytosine, 3-aminophenyl-imidazole, 2'-O-methyl-adenine, 7-deazadenine, 7-deazaguanine, 4-acetylcytosine, 5-(carboxy-hydroxylmethyl)-uracil, 2'-O-methylcytosine, 5-carboxymethyl-aminomethyl-2-thiouracil, 5-carboxymethylamino-methyluracil, dihydrouracil, 2'-O-methyluracil, 2'-O-methyl-pseudouracil, ß-D-galactosylqueonine, 2'-O-methylguanine, xanthine, hypoxanthine, N6-isopentenyladenine, 1-methyladenine, 1-methyl-pseudouracil, 1-methylguanine, 1-methylxanthine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, 5-methyluracil, N6-methyl-adenine, 7-methylguanine, 5-methylamino-methyluracil, 5-methoxyaminomethyl-2-thiouracil, ß-D-mannosylqueonine, 5-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methyl-thio-N6-isopentenyladenine, N-(9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)-carbamoyl)threonine, N-(9-beta-D-ribofuranosylpurine-6-yl)-N-methylcarbamoyl)threonine. B groups in an α-anomeric configuration can also be present in the nucleotides linked by the present methods.

Preferred B groups are unmodified G, A, T, C and U bases. In addition, preferred B groups include pyrimidines and purines with 1-2 substituents independently selected from the group consisting of amino, oxo, hydroxy, lower alkyl, lower alkoxy, lower alkylamine, phenyl or lower alkyl substituted phenyl groups. It is more preferred that these groups are present on the 5 position of the pyrimidine and on the 7 or 8 position of the purine. Especially preferred base analogs are 5-methylcytosine, 5-methyluracil and diaminopurine.

Moreover, the selection of a B group for each nucleotide added to the growing polynucleotide chain determines the nucleotide sequence of an oligonucleotide produced by the present methods. Accordingly, the present methods can be used to generate oligonucleotides having any desired nucleotide sequence by varying which nucleotide base B is placed at each position. The selection of a nucleotide sequence is generally determined by the intended purpose of the oligonucleotide and is described in more detail hereinbelow.

According to the present invention n is an integer used to describe the number of Rp alkyl- or aryl-phosphonate linkages sequentially synthesized by the present methods. As used herein, n is 0 to 200. Moreover, up to 201 Rp alkyl- or aryl-phosphonate linkages can be formed sequentially when n ranges from 0 to is 200. However, when n is 0, a single Rp alkyl- or aryl-phosphonate linkage is formed. Therefore, the present invention is directed towards application of the subject methods to form isolated Rp phosphonate linkages

as well as sequential chains of Rp stereoisomeric alkyl- or aryl-phosphonate linkages.

Preferably, n is at least 5. However, a value of at least 8 is more preferred for n. Even more preferred is a value of at least 10 for n. Especially preferred values for n are at least 12 and 14.

According to the present invention, Y_1 is present on a 3'-oxygen of a nucleotide and can be a hydrogen, phosphate, phosphate present in the oligonucleotide or V_1. V_1 is related to Y_1 in that V_1 and Y_1 are at the same position and Y_1 can have the same meaning as V_1. As used herein V_1 is a protecting group, a solid support or a phosphate attached to a penultimate nucleotide of the oligonucleotide. Such a penultimate nucleotide is the nucleotide next to the 5'-terminal nucleotide.

Moreover, as used herein, Y_2 is present on a 5'-oxygen of a nucleotide or an oligonucleotide and can be a hydrogen, a phosphate, or V_2, wherein V_2 is a protecting group. Since Y_2 and V_2 are at the same position, removal of a V_2 protecting group can generate a Y_2 hydrogen or phosphate.

Similarly, X and V_3 are related not only by virtue of placement at the same position (2') but also because X can have the same meaning as V_3, i.e. X is hydroxy or V_3. When X is V_3, V_3 can be hydrogen or O-Y_3 wherein Y_3 is a lower alkyl or a protecting group. According to the present invention, removal of a Y_3 protecting group can produce a hydroxy group, i.e. X as OH.

As used herein, formulas I and II represent a portion of a oligonucleotide when Y_1 or Y_2 is defined as

a phosphate present in the oligonucleotide. Hence additional nucleotides can flank the Rp phosphonate linkage being formed when Y_1 or Y_2 is a phosphate present in the oligonucleotide. In particular, usage of Y_1 or Y_2 as a phosphate present in the oligonucleotide is intended to indicate that the oligonucleotide can be longer than the n sequential Rp linkages formed according to the present methods. More particularly, the present invention contemplates conventional phosphodiester linkages, or on interspersing of conventional phosphodiester and Rp phosphonate linkages in the parts of the oligonucleotide attached to a Y_1 and Y_2 phosphate. As used herein a conventional phosphodiester linkage is a $-O-PO_2-O-$linkage between 3'- and 5'-positions of two nucleoside sugars.

Preferably about 1 to about 50 $-O-PO_2-O-$ linkages can be added to, or interspersed between, Rp phosphonate linkages of the present oligonucleotides. Moreover, such conventional oligonucleotides are added by known procedures which are readily available to the skilled artisan (e.g., Uhlmann et al. 1990 Chemical Reviews 90: 544-584).

Therefore, the present methods can be adapted to include at least one additional step directed to adding about 1 to about 50 non-alkyl-phosphonate or non-aryl-phosphonate nucleotides wherein such nucleotides are joined by $-O-PO_2-O-$linkages.

As provided by the present invention, an internal or non-terminal Rp linkage is produced when both Y_1 and Y_2 are phosphates present in the oligonucleotide. However, when Y_1 or Y_2 is other than a phosphate present in the oligonucleotide, a 3'-terminal

or a 5'-terminal linkage, respectively, can be made. Accordingly, the present methods can be used to generate both internal and terminal Rp stereoisomeric alkyl- or aryl-phosphonate linkages.

Moreover, sequential Rp linkages can also be formed by the present methods since V_1 can be defined as the phosphate present on the penultimate nucleotide of the oligonucleotide at each round of synthesis. Such a penultimate nucleotide is the nucleotide next to the 5'-terminal nucleotide.

As defined, V_1 can also be a solid support. Preferably V_1 is a solid support when the present methods are performed by automation since V_1 can thereby serve as an anchor for the growing polynucleotide chain. Such a solid support can be any known support used during synthesis of DNA or RNA. Common types of solid supports include controlled pore glass (CPG), polystyrene silica, cellulose, nylon and the like. Preferred solid supports are CPG and polystyrene. An especially preferred solid support is CPG.

The V_1 solid support is covalently linked to the 3'-OH of a nucleoside by known procedures (Matteucci et al. 1980 Tetrahedron Letters 21: 719-722). Alternatively, nucleosides linked to solid supports can be purchased commercially, e.g. from Sigma Chemical Company. Moreover, a solid support can also be removed from an oligonucleotide of the present invention by known procedures, e.g. by alkaline hydrolysis.

The V_1, V_2, V_3 protecting groups can be used when the present synthetic methods are employed to form the subject Rp stereospecific phosphonate linkages. In particular, the present invention provides such

protecting groups for covalent binding to a reactive group on a nucleotide. Such binding by a reactive group can render that reactive group unreactive while the present synthetic methods are performed. Reactive groups of the present invention include 5'-OH, 3'-OH, 2'-OH and related groups, e.g. reactive groups present on the B bases. Ideally, a protecting group is easily removed to regenerate the correct structure of the reactive group without chemically altering the remainder of the molecule.

Examples of protecting groups contemplated by the present invention include any known blocking or protecting agent used during synthesis of deoxyribooligonucleotides or ribooligo-nucleotides to protect a a hydroxy group on a nucleotide, e.g. a 5'-OH, 3'-OH or 2'-OH group. The V_1, V_2 and V_3 protecting groups are preferably attached via an oxygen atom. Such O-linked protecting groups are useful for protecting the OH groups on nucleotides. In this regard, Greene (1981 Protecting Groups in Organic Synthesis, John Wiley & Sons, Inc.) provides a comprehensive review of protecting groups which can be used for different reactive groups including OH reacting groups. Preferred protecting groups are lower alkyl, lower acyl, aroyl, aryloxy, aryloxyacyl, haloaryl, fluorenyl methoxy carbonyl (FMOC), trityl, monomethoxytrityl (MMT), dimethoxytrityl (DMT) and related groups. More preferred protecting groups include isopropyl, isobutyl, 2-cyanoethyl, acetyl, benzoyl, phenoxyacetyl, halophenyl, FMOC, trityl, MMT, DMT and the like.

According to the present invention, an activating group A is an $R\text{-}Z_1\text{-}CO\text{-}$ or $R\text{-}Z_1\text{-}SO_2\text{-}$ wherein:

R is lower alkyl, lower alkenyl, mono-, di- or tri- cycloalkyl, lower carbalkyl, aryl or aryl lower alkyl which can be substituted with up to three lower alkyl, halo, amino, ammonio (NH_4^+) or nitro groups; and

Z_1 is an oxygen atom or a chemical bond.

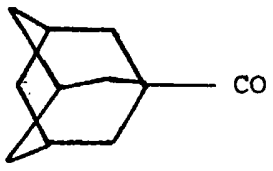
An A activating group of the present invention is preferably a lower alkyl sulfonyl, lower alkyl sulfinyl, lower carbalkyl sulfonyl, lower carbalkyl sulfinyl, lower carbalkoxy, acetyl, lower alkoxy acetyl, benzoyl, adamantoyl, crotonyl or 4-alkoxycrotonyl group, wherein such a lower alkyl, lower carbalkyl, aryl, alkoxy, acetyl, benzoyl, adamantoyl or crotonyl can be substituted with up to three lower alkyl, halo, amino, ammonio or nitro groups.

As used herein a sulfonyl is a SO_3 group. Similarly, a sulfinyl is a SO_2 group. When A includes a sulfonyl or a sulfinyl group, these groups are preferably attached via the sulfur to the 5'-oxygen of the 5'-O-activated nucleotide.

A lower carbalkyl of the present invention is a -CO- attached to a lower alkyl. Similarly, a lower carbalkoxy is a carboxylate (-COO-) with a lower alkyl attached to the monosubstituted carboxylate oxygen.

According to the present invention an acetyl is a $-CO-CH_3$ and a lower alkoxy acetyl is a $-CO-CH_2-O-$ lower alkyl. Moreover, a benzoyl is a benzene with an attached carbonyl.

As used herein an adamantoyl is tricyclohexyl carbonyl of the formula:



Moreover, a crotonyl is a $-CO-CH=CH-CH_3$ and a 4-alkoxycrotonyl group is a $-CO-CH=CH-CH_3$-lower alkyl.

Preferred A groups are lower alkyl sulfonyl, lower alkyl sulfinyl, lower carbalkyl sulfonyl, lower carbalkyl sulfinyl, aryl sulfonyl, aryl sulfinyl, adamantoyl, crotonyl or 4-alkoxycrotonyl groups, wherein such a lower alkyl, lower carbalkyl, aryl, adamantoyl or crotonyl can be substituted with up to three lower alkyl, halo, amino, ammonio or nitro groups.

More preferred A groups are a lower alkyl sulfinyl, aryl sulfinyl, adamantoyl, crotonyl or 4-alkoxycrotonyl group, wherein such a lower alkyl, aryl, adamantoyl or crotonyl can be substituted with up to three lower alkyl, halo, amino, ammonio or nitro groups.

Preferred lower alkyl sulfinyls include methyl sulfinyl (i.e. mesylate), ethyl sulfinyl, propyl sulfinyl, isopropyl sulfinyl, butyl sulfinyl, isobutyl sulfinyl, t-butyl sulfinyl, pentyl sulfinyl, hexyl sulfinyl and the like which are substituted with one ammonio or up to three lower alkyl or halo groups. Especially preferred lower alkyl sulfinyl include methyl sulfinyl (i.e. mesylate), ethyl sulfinyl, propyl sulfinyl and isopropyl sulfinyl which are substituted with three lower alkyl or halo groups, or ammonio-alkylsulfonyl (i.e. betylate). When a lower alkyl sulfinyl has one or more halo substituent the halo is preferably a fluoro.

Preferred lower fluoroalkylsulfinyls include a trifluoromethylsulfinyl (i.e. $-SO_2CF_3$ or triflate), nonafluorobutylsulfinyl (i.e. $SO_2-C_4F_9$ or nonaflate) and 2,2,2-trifluoroethyl-sulfonate (i.e. lower alkyl $-SO_2-CH_2-CH_2CF_3$ or tresylate).

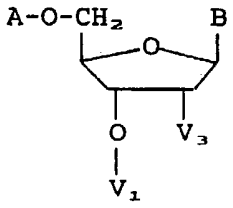
2,2,2-trifluoroethyl-sulfonate (i.e. lower alkyl -SO_2-CH_2-CH_2CF_3 or tresylate).

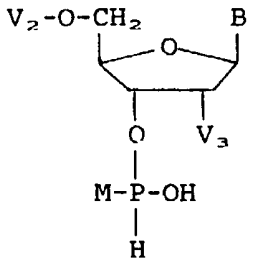
Moreover, preferred aryl sulfinyls include groups such as tolylsulfinyls (i.e. tosylates), and bromophenylsulfinyls (i.e. brosylates), nitrophenyl-sulfinyls (i.e. nosylates) and the like. An especially preferred A group is a lower fluoroalkyl-sulfinyl. The most preferred A group is trifluoromethylsulfinyl, i.e. triflate.

As used herein A, when free from the 5'-O-activated nucleotide is negatively charged and has an attached oxygen atom, i.e. $A-O^-$. Accordingly the present invention contemplates providing $A-O^-$ as a salt. Such $A-O^-$ salts include the negatively charged $A-O^-$ group associated with a cation. Preferred cations are transition metals such as Mn, Co, Ni, Cu, Zn, Mo, Ag, Pt, Au and the like. A preferred cation is Ag.

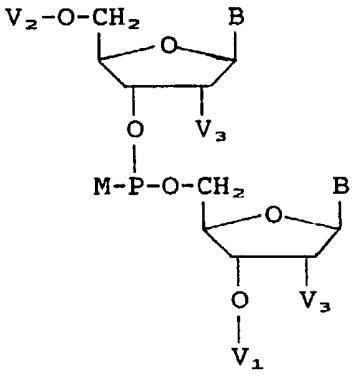
The $A-O^-$ salts of the present invention are either commercially available or are synthesized by available procedures.

According to the present invention, Rp stereoisomeric alkyl- or aryl-phosphonate linkages between any two nucleotides are formed by reacting a 5'-O-activated nucleotide of the formula:





under conditions sufficient to produce an Sp stereoisomeric alkyl- or aryl-phosphonate linkage of the formula:



wherein:

intermediate has Sp phosphorus stereoisomeric configuration;

V_1 is a protecting group, solid support or phosphate present on the penultimate nucleotide of the oligonucleotide;

V_2 is a protecting group;

V_3 is a hydrogen or $O-Y_3$, wherein Y_3 is a lower alkyl or a protecting group; and

M, B and A are as described hereinabove.

As used herein conditions sufficient to produce an Sp stereoisomeric alkyl- or aryl-phosphonate linkage include a time, a temperature, solvent or reactant concentration sufficient for nucleophilic displacement of the 5'-activated oxygen by a phosphate oxygen on the intermediate. Therefore, A-O$^-$ is lost and a covalent bond is formed between the 5' carbon and phosphinate oxygen present on the intermediate.

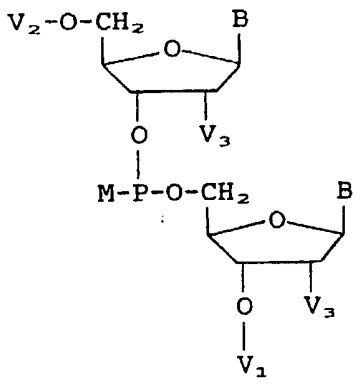
A time sufficient for nucleophilic displacement is about 10 sec to about 1 hr and preferably about 1 min to about 10 min.

Moreover a temperature sufficient for nucleophilic displacement is about 4°C to about 50°C and preferably about 20°C to about 25°C.

A solvent which is used by the present invention for nucleophilic displacement is an anhydrous solvent and is preferably a nonpolar or nonpolar aprotic solvent such as tetrahydrofuran, dimethylsulfoxide, pyridine, dimethylformamide, acetonitrile and the like.

Furthermore a reactant concentration sufficient for nucleophilic displacement is a molar ratio of 5'-O-activated nucleotide to intermediate ranging from 1:100 to about 1:1. A preferred molar ratio is about 1:10.

Furthermore the present methods are directed to inverting the configuration of the Sp stereoisomeric alkyl- or aryl-phosphonate linkage depicted below:



by reacting the linkage with an oxidizing agent under conditions sufficient to produce the Rp stereoisomeric alkyl- or aryl-phosphonate linkage.

According to the present invention, conditions sufficient to produce such a Rp stereoisomeric alkyl- or aryl-phosphonate linkage include a time, a solvent, a temperature and an oxidizing agent concentration sufficient for oxidation, and inversion of the Sp configuration of such a Sp stereoisomeric alkyl- or aryl-phosphonate.

As used herein a time sufficient for such oxidation and inversion of the Sp linkage is about 1 min to about 60 min and preferably about 5 min.

Moreover a solvent sufficient for oxidation and inversion is an aqueous solvent, preferably water.

A temperature for oxidation and inversion of the Sp linkage is about 4°C to about 50°C and preferably about 20°C to about 25°C.

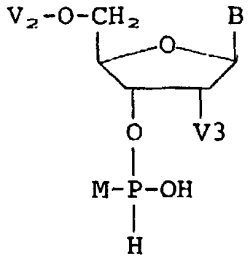
Furthermore an oxidizing agent concentration sufficient for oxidation and inversion of the Sp linkage is a molar ratio of oxidizing agent to Sp linkage of about 100:1 to about 1:1. Preferably such a molar ratio

of oxidizing agent to Sp linkage is about 10:1 to about 1:1. An especially preferred molar ratio is about 2:1.

Oxidizing agents for preparation of the present Rp stereoisomeric alkyl- or aryl-phosphonate linkages include any agent capable of forming a phosphonate (O-P[M]O-O) from a phosphinate (O-PM-O). The oxidizing agents contemplated by the present invention are mild oxidizing agents which will not oxidize any of the B groups substituents, such as a halogen, peracid, peralkanoic acid, e.g., peracetic acid, ozone, hydrogen peroxide, and the like. Preferred oxidizing agents include but are not limited to halogens, e.g. I_2/H_2O.

In one especially preferred embodiment, the present methods are performed automatically in a nucleic acid synthesizer. The present methods have been designed for adaptation to automation by selecting reactions which can be performed under conditions typically used in nucleic acid synthesizers. For example, the temperatures, solvents and reagents contemplated herein are compatible with procedures and common protecting agents employed during automated nucleic acid synthesis (see Uhlmann et al. 1990 for a review of such procedures). Accordingly, adaptation of the present methods to automation is readily accomplished by one of skill in the art.

In another embodiment, the present invention is directed to an alkyl- or aryl-phosphinate nucleotide intermediate which has an Sp stereoisomeric configuration at the phosphorus. This intermediate is of the formula:



wherein V_2, B, V_3 and M are as defined hereinabove.

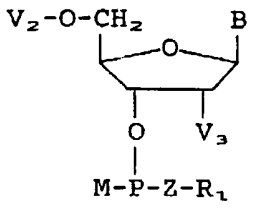
Preferred B groups for the present intermediates include pyrimidines and purines with 1-2 amino, oxo, hydroxy lower alkyl, lower alkoxy, lower alkylamine, phenyl or lower alkyl substituted phenyl groups. In a more preferred embodiment, the alkyl- or aryl-phosphinate nucleotide intermediate has a B group selected from the group of guanine, adenine, thymine, cytosine or uracil.

Moreover, the intermediate preferably has an M group which is lower alkyl or aryl. An especially preferred M group on the intermediate is methyl or ethyl.

As used herein, V_3 is preferably hydrogen. However, V_3 can also be $O-Y_3$, in which case Y_3 is preferably a protecting group.

Furthermore, the intermediate preferably has dimethoxytrityl or monomethoxytrityl V_2 or Y_3 protecting groups.

The intermediate is formed by hydrating a racemic phosphono-nucleotide of the formula:



in the presence of an effective amount of a hydrating catalyst under conditions sufficient for forming a racemic phosphinate nucleotide;

wherein:

V_2, B, M, and V_3 are as described hereinabove;

Z is -S- or $-NR_2$; and

R_1 and R_2 are independently lower alkyl, lower alkenyl, or R_1 and R_2 are taken together with the nitrogen to which they are attached to form a 5 or 6 membered heterocyclic or heteroaromatic ring.

According to the present invention Z is -S- or $-NR_2$, however, $-NR_2$ is a preferred Z group. Therefore, in a preferred embodiment the racemic phosphononucleotide is a phosphonoamidite wherein both R_1 and R_2 are present, i.e. as $-NR_2R_1$. Moreover, as used herein R_1 and R_2 are independently lower alkyl, lower alkenyl, or R_1 and R_2 are taken together with the nitrogen to which they are attached to form a 5 or 6 membered heterocyclic or heteroaromatic ring.

While the R_1 and R_2 lower alkyl and lower alkenyl groups can have 1-6 carbons, preferred R_1 and R_2 groups have at least two carbon atoms and more preferably have at least three carbon atoms. Accordingly preferred R_1 and R_2 lower alkyl groups are ethyl, propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, pentyl, isopentyl, hexyl and the like.

Similarly, preferred lower alkenyl groups have 2-6 carbon atoms, and additionally have 1-3 carbon-carbon double bonds. Moreover, the lower alkyl and alkenyl groups of the present invention are preferably branched, e.g. isopropyl, isobutyl, sec-butyl, tert-butyl, isopentyl, neopentyl and the like. An especially preferred R_1 or R_2 lower alkyl is isopropyl.

Moreover according to the present invention, R_1 and R_2 can be taken together with the nitrogen to which they are attached to form a 5 or 6 membered heterocyclic ring. As used herein, a heterocyclic ring includes saturated, partially saturated and heteroaromatic rings. Moreover, heterocyclic groups of the present invention are either monocyclic or bicyclic with at least one ring nitrogen heteroatom and 5 to 10 ring atoms. Heterocyclic rings can also have at least one other nitrogen, sulfur or oxygen ring atom. More preferred heterocyclic rings have 1-3 nitrogen ring atoms and can also have 1 oxygen ring atom. Especially preferred heterocyclic rings are monocyclic with 5 or 6 ring atoms and one nitrogen heteroatom.

R_1 and R_2 heterocyclic rings, as contemplated by the present invention, include piperidine, morpholine, piperazine, pyrrole, pyrrolidine, isopyrrole, pyrazole, imidazole, isoimidazole, triazole, oxazole, isoxazole, thiazole, isothiazole, oxodiazole, tetrazole, pyrazine, pyridazine, pyrimidine, pyridine, oxazine, isoxazine, oxadiazine, imidazole, indole, pyridine, quinoline, isoquinoline, pyridopyridine, purine and the like.

Preferred R_1 and R_2 heterocyclic and heteroaromatic rings include piperidine, morpholine,

imidazole, pyrrole, pyrrolidine, pyridine, pyrimidine, triazole, tetrazole, indole, pyridopyridine rings and the like. More preferred R_1 and R_2 heterocyclic rings are piperidine, morpholine, pyrrolidine, imidazole, imidazolidine, pyrrole, pyridine, pyrimidine, triazole and tetrazole. Especially preferred heterocyclic rings are piperidine, morpholine, pyrrolidine, imidazole or triazole.

Moreover, as used herein a catalyst for hydrating the phosphono-nucleotide is a heterocyclic ring which can displace the Z group and subsequently be replaced by a water OH. Such a catalyst heterocyclic ring, preferably has up to 4 nitrogen ring heteroatoms and can also have up to three lower alkyl substituents. According to the present invention, catalyst heterocyclic rings include pyrazole, imidazole, isoimidazole, triazole, oxadiazole, pyridazine, pyrimidine, pyrazine, piperazine, triazine, tetrazole and the like which can have up to three lower alkyl substituents. When an alkyl substituent is present, such an alkyl is preferably present on a nitrogen heteroatom. Preferred hydration catalysts are N-heterocyclic rings which can have up to two lower alkyl substituents. Such preferred hydration catalysts include tetrazole, triazole, N-alkyl imidazole and the like. An especially preferred hydration catalyst is tetrazole.

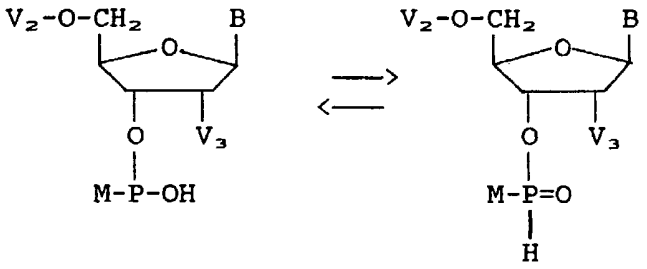
Conditions sufficient for forming a racemic phosphinate nucleotide from the racemic phosphono-nucleotide include a time, temperature, solvent and hydrating catalyst concentration sufficient for displacement of Z by a water OH.

A time for hydration is about 1 sec to about 10 min, or preferably is about 1 min.

A preferred temperature for hydration of a phosphononucleotide is about 4°C to about 42°C. A more preferred temperature is about room temperature, i.e. about 20°C to about 25°C.

A solvent for hydration is preferably water, and a hydration catalyst concentration is a molar ratio of catalyst to phosphono-nucleotide of about 20:1 to about 1:1. A preferred ratio is about 10:1 to about 2:1 and a more preferred ratio is about 5:1.

Hydration of the phosphono-nucleotide generates the intermediate which can tautomerize between two forms. According to the present invention, tautomerization does not alter the stereisomeric configuration of the phosphorus. The two tautomeric forms of the intermediate are of the formula:



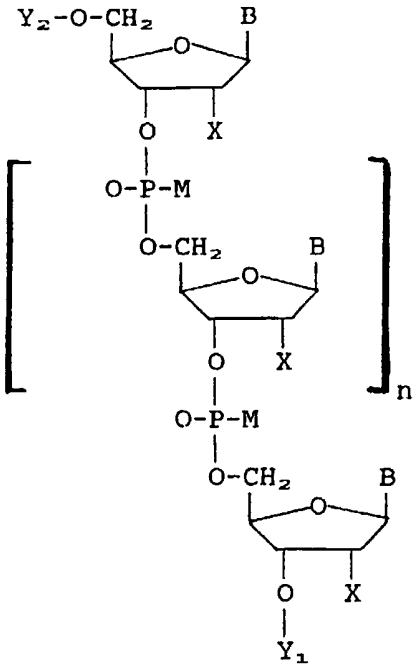
wherein:

V_2, B, M, and V_3 are as described hereinabove.

The phosphono-nucleotide has a racemic phosphorus which remains racemic during hydration. However, according to the present invention, the Rp and Sp stereoisomers of the intermediate are stable and can

be chromatographically separated. Separation of Rp and Sp stereoisomers of phosphonate nucleotides is known (Miller et al. 1979 Biochemistry 18: 5134; and Lebedev, et al. 1990c Tetrahedron Letters 31: 3673-3676). Any type of chromatographic medium useful for stereoisomeric separation is contemplated by this invention, including high pressure liquid chromatography (HPLC) and non-HPLC chromatographic procedures. Moreover, stereoisomers of the present phosphonate nucleotides can be separated by both reversed phase and normal phase chromatography (Lebedev, et al. 1990a Tetrahedron Letters 31: 851-854; and Lebedev, et al. 1990c). In a preferred embodiment, the Rp and Sp stereoisomers are separated by either normal or reversed phase HPLC using a silica gel, or C_{18} gel matrix. When using normal phase HPLC the silica gel can be pre-treated with base, for example a trialkylamine such as triethylamine. The stereoisomers are then eluted by a using a small amount of a polar solvent, e.g. ethanol, in a non-polar solvent, e.g. chloroform. When using reverse phase HPLC the stereoisomers can be separately eluted from silica gel by using a small amount of non-polar solvent, e.g. acetonitrile, in a polar solvent, e.g. water.

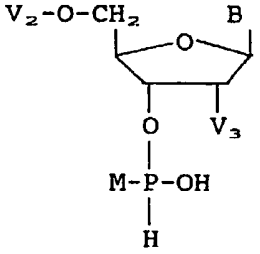
In another embodiment, the present invention is directed to a compartmentalized kit for producing a polynucleotide chain of an oligonucleotide having at least five sequential R-alkyl-phosphonate or R-aryl-phosphonate linkages, wherein the oligonucleotide has the formula:



wherein Y_1, Y_2, X, M and B are defined as hereinabove; and n is 4-200. Such a kit can include:

(a) a first container adapted to contain a salt of an $A\text{-}O^-$ activator; and

(b) a second container adapted to contain a first alkyl- or aryl-phosphinate nucleotide intermediate of the formula:



wherein V_2, B, V_3 and M are as defined hereinabove.

Moreover, the kit can be further adapted to contain at least one additional container containing a second alkyl- or aryl-phosphinate nucleotide intermediate, wherein the second intermediate has an Sp stereoisomeric phosphorus configuration and a different B group than the first intermediate.

In a preferred embodiment, the first or second alkyl- or aryl- phosphinate nucleotide intermediate provided in the kit has a B group selected from the group of guanine, adenine, thymine, cytosine or uracil.

Moreover, when an intermediate is provided in a kit the M group thereupon is preferably lower alkyl or aryl. A more preferred M group is methyl or ethyl.

In addition, a preferred V_2 or Y_3 protecting group for an intermediate provided in a kit of the present invention is dimethoxytrityl or monomethoxytrityl.

Furthermore, the present kits preferably have salts of the preferred activator $A\text{-}O^-$, described hereinabove, e.g. a salt of fluoroalkylsulfonate. Preferred salts of fluoroalkylsulfonates are silver salts of trifluoromethylsulfonate, nonafluorobutyl-sulfonate or 2,2,2-trifluoroethylsulfonate. An especially preferred salt of $A\text{-}O^-$ is silver trifluoromethylsulfonate.

In a more preferred embodiment the kit provides a first container containing a salt of an $A\text{-}O^-$, a second container containing a salt of alkyl- or aryl-phosphonothioate guanine, a third container containing a salt of alkyl- or aryl-phosphonothioate adenine, a fourth container containing a salt of alkyl- or aryl-phosphonothioate cytosine, a fifth container containing

a salt of alkyl- or aryl-phosphonothioate thymine and optionally a sixth container containing a salt of alkyl- or aryl-phosphonothioate uracil(?).

As used herein, salts of the present alkyl- or aryl-phosphonothioate nucleotide intermediate are alkali metal or alkaline earth metal salts, for example Li, Na, K, Mg, Ca, and the like. Preferred salts are alkali metal salts, e.g., Li, Na, and K. Especially preferred salts are Li salts.

After synthesis by the present methods an oligonucleotide can be purified by polyacrylamide gel electrophoresis, or by any of a number of chromatographic methods, including gel chromatography and high pressure liquid chromatography.

In a preferred embodiment the present invention is directed to an oligonucleotide having at least five sequential Rp stereospecific alkyl- or aryl-phosphonate linkages produced by the present methods.

While the oligonucleotides prepared by the present methods can have as little as five sequential Rp stereospecific alkyl- or aryl-phosphonate linkages, preferred oligonucleotides have more than five Rp stereospecific linkage. For example, oligonucleotides synthesized by the methods of the present invention generally have about 8 to about 200 alkyl- or aryl-phosphonate linkages. Preferred oligonucleotides of the present invention have about 10 to about 200 alkyl- or aryl-phosphonate linkages. More preferred oligonucleotides have about 12 to about 200 alkyl- or aryl-phosphonate linkages. Especially preferred oligonucleotides of the present invention have about 14 to about 200 alkyl- or aryl-phosphonate linkages.

According to the present invention, the subject methods produce Rp stereospecific linkages at a higher frequency than Sp stereospecific linkages. However, not all of the alkyl- or aryl-phosphonate linkages produced by the present methods may be Rp stereospecific. Therefore, Sp stereospecific linkages can occasionally be produced, for example, if the preparation of alkyl- or aryl-phosphonothioate nucleotide precursors employed have a small percentage of Rp stereoisomeric nucleotide contaminants. Accordingly, the present invention is directed to methods of producing a higher percentage of Rp stereospecific alkyl- and aryl-phosphonate linkages than Sp stereospecific alkyl- and aryl-phosphonate linkages.

In particular the present methods can produce at least about 75% Rp stereospecific linkages in an oligonucleotide wherein the remaining linkages can be Sp stereospecific. More particularly, the oligonucleotides generated by the present methods have about 85% to about 100% Rp stereospecific linkages. However, the present methods have the capability for producing oligonucleotides having about 95% to 100% Rp stereospecific alkyl- or aryl-phosphonate linkages.

Moreover, the oligonucleotides of the present invention need not have only alkyl- or aryl-phosphonate linkages. In some instances oligonucleotides having a mixture of conventional phosphodiester linkages (-O-PO_2-

O) and phosphonate (-O-PO-O-) linkages are preferred.
|
M

For example, conventional phosphodiester linkages may be incorporated into the present oligonucleotides to generate an endonuclease cleavage site or to render the oligonucleotide sensitive to normal cellular enzymes at a particular sequence within the oligonucleotide. If the subject oligonucleotides have conventional phosphodiester linkages these oligonucleotides can have about 1 to about 50 conventional phosphodiester linkages.

Therefore, the present invention is directed to oligonucleotides which can have conventional phosphodiester linkages, as well as both Sp stereospecific and Rp stereospecific phosphonate linkages, so long as the oligonucleotide has at least five, and preferably eight to fourteen, sequential Rp stereospecific alkyl- or aryl-phosphonate linkages generated by the present methods.

In a preferred embodiment, the oligonucleotides produced by the present methods have B groups which include pyrimidines and purines with 1-2 amino, oxo, hydroxy, lower alkyl, lower alkoxy, lower alkylamine, phenyl or lower alkyl substituted phenyl groups. Moreover, preferably, B is selected from the group of guanine, adenine, thymine, cytosine or uracil.

Moreover, the present oligonucleotides preferably have M as lower alkyl or aryl. A more preferred M group is methyl or ethyl.

The preferred Y_1 and Y_2 groups for the present oligonucleotides are hydrogen, phosphate or phosphate attached to the oligonucleotide. Preferred X groups of

the present oligonucleotides are hydroxy and V_3, wherein V_3 is hydrogen.

Moreover, according to the present invention, Rp stereospecific oligonucleotide products derived from the subject synthetic methods can have an attached agent to facilitate cellular delivery or uptake. Such an agent can, for example, be any known moiety which enhances cellular membrane penetration by the oligonucleotide, any known ligand for a cell-specific receptor or any availible antibody reactive with a cell-specific antigen.

A moiety or ligand which enhances cellular membrane penetration by the oligonucleotide can include, for example, any non-polar group, steriod, hormone, polycation, protein carrier, or viral or bacterial protein capable of cell membrane penetration. Such a non-polar group can be a phenyl, naphthyl, quinoline, anthracene, phenanthracene, fatty acid, fatty alcohol, sesquiterpene, diterpene and related groups. Steroids which can enhance cell uptake include cholesterol, progesterone, estrogen, androgen and related steroids. For example, covalent linkage of a cholesterol moiety to an oligonucleotide can improve cellular uptake by 5- to 10- fold which in turn improves DNA binding by about 10-fold (Boutorin et al., 1989, FEBS Letters 254: 129-132). Hormones such as insulin can also bind to cell membranes and facilitate entry of an oligonucleotide thereto into the cell. Polycations, e.g. polyamino acid cations, including cations of basic amino acids, such as poly-L-lysine, can also facilitate uptake of oligonucleotides into cells (Schell, 1974, Biochem. Biophys. Acta 340: 323, and Lemaitre et al., 1987,

Proc. Natl. Acad. Sci. USA 84: 648). Certain protein carriers can also facilitate cellular uptake of oligonucleotides, including, for example, serum albumin, transferrin, nuclear proteins possessing signals for transport to the nucleus, and viral or bacterial proteins capable of cell membrane penetration. Accordingly, the present invention contemplates derivatization of the subject oligonucleotides with the above-identified groups to increase oligonucleotide cellular uptake.

Moreover, the present invention contemplates the preparation of Rp stereospecific linkages in oligonucleotides having any nucleotide sequence. In many instances the selection of a nucleotide sequence depends upon the intended purpose of the oligonucleotide, for example the nucleotide sequence can be selected for the purpose of binding to a nucleic acid target. Such a nucleic acid target can be present within a template nucleic acid which encodes a DNA, RNA or protein. Moreover, binding of the subject oligonucleotides can be used, for example, to detect or to regulate the biosynthesis of such a template nucleic acid.

The present invention contemplates a variety of utilities for the subject Rp stereospecific oligonucleotides. Some utilities include, but are not limited to: use of oligonucleotides of defined sequence bound to a solid support for affinity isolation of complementary nucleic acids; covalent attachment of a drug, drug analog or other therapeutic agent to the oligonucleotide to allow cell-type specific drug delivery; labeling the subject oligonucleotides with a

detectable reporter molecule for localizing, quantitating or identifying complementary target nucleic acids; and binding oligonucleotides to a cellular or viral nucleic acid template and regulating biosynthesis directed by that template.

The subject oligonucleotides can be attached to a solid support such as silica, cellulose, nylon, polystyrene, polyethylene glycol, Sepharose 4B^{R} and other natural or synthetic materials that are used to make beads, filters, and column chromatography resins. Attachment procedures for nucleic acids to solid supports of these types are well known; any known attachment procedure is contemplated by the present invention. An oligonucleotide attached to a solid support can then be used to isolate a complementary nucleic acid. Isolation of the complementary nucleic acid can be done by incorporating the oligonucleotide:solid support into a column for chromatographic procedures. Other isolation methods can be done without incorporation of the oligonucleotide:solid support into a column, e.g. by utilization of filtration procedures. Oligonucleotide:solid supports can be used, for example, to isolate poly(A)$^{+}$ mRNA from total cellular or viral RNA by making an Rp alkyl- or aryl-phosphonate oligonucleotide with only poly(dT) or poly(U) B groups. The present Rp alkyl- and aryl-phosphonate oligonucleotides are ideally suited to applications of this type because they are nuclease resistant and bind strongly to target nucleic acids.

The present invention also contemplates using the subject oligonucleotides for targeting drugs to

specific cell types. Such targeting can allow selective destruction or enhancement of particular cell types, e.g. inhibition of tumor cell growth can be attained. Different cell types express different genes, so that the concentration of a particular mRNA can be greater in one cell type relative to another cell type, such an mRNA is a target mRNA for cell type specific drug delivery by oligonucleotides linked to drugs or drug analogs. Cells with high concentrations of target mRNA are targeted for drug delivery by administering to the cell an oligonucleotide with a covalently linked drug that is complementary to the target mRNA.

The present invention also contemplates labeling the subject oligonucleotides for use as probes to detect a target nucleic acid. Labelled oligonucleotide probes have utility in diagnostic and analytical hybridization procedures for localizing, quantitating or detecting a target nucleic acid in tissues, chromosomes or in mixtures of nucleic acids. Oligonucleotide probes of this invention represent a substantial improvement over conventional nucleic acid probes for such procedures because the present Rp stereospecific linkages provide oligonucleotides with increased binding stability.

Labeling an oligonucleotide can be done by incorporating nucleotides linked to a "reporter molecule" into the subject oligonucleotides. A "reporter molecule", as defined herein, is a molecule or atom which, by its chemical nature, provides an identifiable signal allowing detection of the oligonucleotide. Detection can be either qualitative or quantitative. The present invention contemplates using

any commonly used reporter molecule including radionuclides, enzymes, biotins, psoralens, fluorophores, chelated heavy metals, and luciferin. The most commonly used reporter molecules are either enzymes, fluorophores or radionuclides which can be linked to nucleotides either before or after oligonucleotide synthesis. Preferably, the reporter molecule is added after oligonucleotide synthesis, for example, by forming a covalent linkage between a 3'- or 5'-terminal hydroxy or phosphate and a phosphate, nitrogen, sulfor or oxygen atom on the reporter molecule.

Commonly used enzymes include horseradish peroxidase, alkaline phosphatase, glucose oxidase and ß-galactosidase, among others. The substrates to be used with the specific enzymes are generally chosen because a detectably colored product is formed by the enzyme acting upon the substrate. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for horseradish peroxidase, 1,2-phenylenediamine, 5-aminosalicyclic acid or toluidine are commonly used.

The probes so generated have utility in the detection of a specific DNA or RNA target in, for example, Southern analysis, Northern analysis, in situ hybridization to tissue sections or chromosomal squashes and other analytical and diagnostic procedures. Methods of using such hybridization probes are well known and examples of such methodology are provided by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Press, NY).

The present oligonucleotides can be used in conjunction with any known detection or diagnostic procedure which is based upon hybridization of a probe to a target nucleic acid. Moreover, the present oligonucleotides can be used in any hybridization procedure which quantitates a target nucleic acid, e.g., by competitive hybridization between a target nucleic acid present in a sample and a labeled tracer target for one of the present oligonucleotides. Furthermore, the reagents needed for making a oligonucleotide probe and for utilizing such a probe in a hybridization procedure can be marketed in a kit.

The kit for detection of a hybridized oligonucleotide probe of the present invention can be compartmentalized for ease of utility and can contain at least one first container providing an oligonucleotide of the present invention. The kit can also be adapted to contain at least one other container providing reagents for labeling the oligonucleotide with a reporter molecule. Moreover, the kit can be further adapted to contain at least one other container providing reagents for detecting the reporter molecule linked to the oligonucleotide.

Moreover the present invention provides a kit for isolation of a template nucleic acid. Such a kit has at least one first container providing one of the present oligonucleotides which is complementary to a target contained within the template. For example, the template nucleic acid can be cellular and/or viral poly(A)$^+$ mRNA and the target can be the poly(A)$^+$ tail. Hence oligonucleotides of the present invention which

have utility for isolation of poly(A)+ mRNA have a nucleotide sequence of poly(dT) or poly(U).

Furthermore, the present invention provides kits useful when diagnosis of a disease depends upon detection of a specific, known target nucleic acid. Such nucleic acid targets can be, for example, a viral nucleic acid, an extra or missing chromosome or gene, a mutant cellular gene or chromosome, an aberrantly expressed RNA and others. Examples of such target nucleic acids contemplated by the present invention are provided hereinbelow.

These diagnostic kits can be compartmentalized to contain at least one first container providing a oligonucleotide linked to a reporter molecule and can contain at least one second container providing reagents for detection of the reporter molecule.

One aspect of the present invention provides a method of regulating biosynthesis of a DNA, an RNA or a protein by contacting at least one of the subject oligonucleotides with a nucleic acid template for that DNA, that RNA or that protein in an amount and under conditions sufficient to permit the binding of the oligonucleotide(s) to a target sequence contained in the template. The binding between the oligonucleotide(s) and the target can regulate biosynthesis of the nucleic acid or the protein, e.g. by blocking access to the template. When access to the template is blocked proteins and nucleic acids involved in the biosynthetic process are prevented from binding to the template, from moving along the template, or from recognizing signals encoded within the template.

As used herein, biosynthesis of a nucleic acid or a protein includes cellular and viral processes such as DNA replication, DNA reverse transcription, RNA transcription, RNA splicing, RNA polyadenylation, RNA translocation and protein translation, and related processes which can lead to production of DNA, RNA or protein, and involve a nucleic acid template at some stage of the biosynthetic process.

As used herein, a nucleic acid template can be an RNA or a DNA template.

As contemplated by the present invention, regulating biosynthesis includes inhibiting, stopping, increasing, accelerating or delaying biosynthesis. Regulation may be direct or indirect, i.e. biosynthesis of a DNA, RNA or protein may be regulated directly by binding a oligonucleotide to the template for that DNA, RNA or protein; alternatively, biosynthesis may be regulated indirectly by oligonucleotide binding to a second template encoding a protein that plays a role in regulating the biosynthesis of the first DNA, RNA or protein.

DNA replication from a DNA template is mediated by proteins which bind to an origin of replication where they open the DNA and initiate DNA synthesis along the DNA template. To inhibit DNA replication in accordance with the present invention, oligonucleotides are selected which bind to one or more targets in an origin of replication. Such binding blocks template access to proteins involved in DNA replication. Therefore initiation and procession of DNA replication is inhibited. As an alternative method of inhibiting DNA replication, expression of the proteins

which mediate DNA replication can be inhibited at, for example, the transcriptional or translational level.

DNA replication from an RNA template is mediated by reverse transcriptase binding to a region of RNA also bound by a nucleic acid primer. To inhibit DNA replication from an RNA template, reverse transcriptase or primer binding can be blocked by binding a oligonucleotide to the primer binding site, and thereby blocking access to that site. Moreover, inhibition of DNA replication can occur by binding a oligonucleotide to a site residing in the RNA template since such binding can block access to that site and to downstream sites, i.e. sites on the 3' side of the target or binding site.

To initiate RNA transcription, RNA polymerase recognizes and binds to specific start sequences, or promoters, on a DNA template. Binding of RNA polymerase opens the DNA template. There are also additional transcriptional regulatory elements that play a role in transcription and are located on the DNA template. These transcriptional regulatory elements include enhancer sequences, upstream activating sequences, repressor binding sites and others. All such promoter and transcriptional regulatory elements, singly or in combination, are targets for the subject oligonucleotides. Oligonucleotide binding to these sites can block RNA polymerase and transcription factors from gaining access to the template and thereby regulating, e.g., increasing or decreasing, the production of RNA, especially mRNA and tRNA. Additionally, the subject oligonucleotides can be targeted to the coding region or 3'-untranslated region

of the DNA template to cause premature termination of transcription. One skilled in the art can readily design oligonucleotides for the above target sequences from the known sequence of these regulatory elements, from coding region sequences, and from consensus sequences.

RNA transcription can be increased by, for example, binding a oligonucleotide to a negative transcriptional regulatory element or by inhibiting biosynthesis of a protein that can repress transcription. Negative transcriptional regulatory elements include repressor sites or operator sites, wherein a repressor protein binds and blocks transcription. Oligonucleotide binding to repressor or operator sites can block access of repressor proteins to their binding sites and thereby increase transcription.

The primary RNA transcript made in eukaryotic cells, or pre-mRNA, is subject to a number of maturation processes before being translocated into the cytoplasm for protein translation. In the nucleus, introns are removed from the pre-mRNA in splicing reactions. The 5' end of the mRNA is modified to form the 5' cap structure, thereby stabilizing the mRNA. Various bases are also altered. The polyadenylation of the mRNA at the 3' end is thought to be linked with export from the nucleus. The subject oligonucleotides can be used to block any of these processes.

A pre-mRNA template is spliced in the nucleus by ribonucleoproteins which bind to splice junctions and intron branch point sequences in the pre-mRNA. Consensus sequences for 5' and 3' splice junctions and for the intron branch point are known. For example,

inhibition of ribonucleoprotein binding to the splice junctions or inhibition of covalent linkage of the 5' end of the intron to the intron branch point can block splicing. Maturation of a pre-mRNA template can, therefore, be blocked by preventing access to these sites, i.e. by binding oligonucleotides of this invention to a 5' splice junction, an intron branch point or a 3' splice junction. Splicing of a specific pre-mRNA template can be inhibited by using oligonucleotides with sequences that are complementary to the specific pre-mRNA splice junction(s) or intron branch point. In a further embodiment, a collection of related splicing of pre-mRNA templates can be inhibited by using a mixture of oligonucleotides having a variety of sequences that, taken together, are complementary to the desired group of splice junction and intron branch point sequences.

Polyadenylation involves recognition and cleavage of a pre-mRNA by a specific RNA endonuclease at specific polyadenylation sites, followed by addition of a poly(A) tail onto the 3' end of the pre-mRNA. Hence, any of these steps can be inhibited by binding the subject oligonucleotides to the appropriate site.

RNA translocation from the nucleus to the cytoplasm of eukaryotic cells appears to require a poly(A) tail. Thus, a oligonucleotide is designed in accordance with this invention to bind to the poly(A) tail and thereby inhibit RNA translocation. The sequence of such an oligonucleotide can consist of about 10 to about 50 thymine residues, and preferably about 20 thymine residues.

Protein biosynthesis begins with the binding of ribosomes to an mRNA template, followed by initiation and elongation of the amino acid chain via translational "reading" of the mRNA. Protein biosynthesis, or translation, can thus be blocked or inhibited by blocking access to the template using the subject oligonucleotides to bind to targets in the template mRNA. Such targets contemplated by this invention include the ribosome binding site the 5' mRNA cap site, the initiation codon, a site between a 5' mRNA cap site and the initiation codon and sites in the protein coding sequence. There are also classes of protein which share domains of nucleotide sequence homology. Thus, inhibition of protein biosynthesis for such a class can be accomplished by targeting the homologous protein domains (via the coding sequence) with the subject oligonucleotides.

Regulation of biosynthesis by any of the aforementioned procedures has utility for many applications. For example, genetic disorders can be corrected by inhibiting the production of mutant or over-produced proteins, or by increasing production of under-expressed proteins; the expression of genes encoding factors that regulate cell proliferation can be inhibited to control the spread of cancer; and virally encoded functions can be inhibited to combat viral infection.

Some types of genetic disorders that can be treated by the oligonucleotides of the present invention include Alzheimer's disease, some types of arthritis, sickle cell anemia, and types of cancer for which patients can be a genetically predisposed, as well as

other genetic disorders. Many types of viral infections can be treated by utilizing the oligonucleotides of the present invention, including infections caused by influenza, rhinovirus, human immunovirus, herpes simplex, papilloma virus, cytomegalovirus, Epstein-Barr virus, adenovirus, vesticular stomatitus virus, rotavirus and respitory synctitial virus among others. According to the present invention, animal and plant viral infections may also be treated by administering the subject oligonucleotides.

Accordingly, template nucleic acids contemplated by the present invention include cellular oncogenes, genes having a role in Alzheimer's disease, genetic functions encoded by viruses such as those described above, and others. Such template nucleic acids include but are not limited to SEQ ID NO:1 to SEQ ID NO:98 which encode the following genetic functions:

SEQ ID NO:1 human c-abl;
SEQ ID NO:2 human c-bcl-2a;
SEQ ID NO:3 human c-bcl-2b;
SEQ ID NO:4 human c-bcr-1;
SEQ ID NO:5 human c-bcr-2;
SEQ ID NO:6 human c-bcr-3;
SEQ ID NO:7 human c-cbl;
SEQ ID NO:8 human c-erbB-2;
SEQ ID NO:9 human c-ets-1;
SEQ ID NO:10 human c-dbl;
SEQ ID NO:11 human c-fgf;
SEQ ID NO:12 human c-fgr-1;
SEQ ID NO:13 human c-fgr-2;
SEQ ID NO:14 human c-fgr-3;
SEQ ID NO:15 human c-fgr-4;

SEQ ID NO:16 human c-fgr-5;
SEQ ID NO:17 human c-fgr-6;
SEQ ID NO:18 human c-fgr-7;
SEQ ID NO:19 human c-fms;
SEQ ID NO:20 human c-fos;
SEQ ID NO:21 human c-has/bas;
SEQ ID NO:22 human c-int-1;
SEQ ID NO:23 human c-int-2;
SEQ ID NO:24 human c-jun;
SEQ ID NO:25 human c-kit;
SEQ ID NO:26 human c-mas;
SEQ ID NO:27 human c-met;
SEQ ID NO:28 human c-myc;
SEQ ID NO:29 human c-Ki-ras1;
SEQ ID NO:30 human N-ras-1;
SEQ ID NO:31 human N-ras-2;
SEQ ID NO:32 human N-ras-3;
SEQ ID NO:33 human N-ras-4;
SEQ ID NO:34 human c-ret;
SEQ ID NO:35 human c-ros-1;
SEQ ID NO:36 human c-ros-2;
SEQ ID NO:37 human c-ros-3;
SEQ ID NO:38 human c-ros-4;
SEQ ID NO:39 human c-ros-5;
SEQ ID NO:40 human c-ros-6;
SEQ ID NO:41 human c-ros-7;
SEQ ID NO:42 human c-ros-8;
SEQ ID NO:43 human c-ros-9;
SEQ ID NO:44 human c-ros-10;
SEQ ID NO:45 human c-sec;
SEQ ID NO:46 human c-sis-1;
SEQ ID NO:47 human c-sis-2;

SEQ ID NO:48 human c-sis-3;
SEQ ID NO:49 human c-sis-4;
SEQ ID NO:50 human c-sis-5;
SEQ ID NO:51 human c-sis-a1;
SEQ ID NO:52 human c-sis-a2;
SEQ ID NO:53 human c-sis-a3;
SEQ ID NO:54 human c-sis-a4;
SEQ ID NO:55 human c-sis-a5;
SEQ ID NO:56 human c-sis-a6;
SEQ ID NO:57 human c-sis-a7;
SEQ ID NO:58 human c-sis-b1;
SEQ ID NO:59 human c-sis-b2;
SEQ ID NO:60 human c-sis-b3;
SEQ ID NO:61 human c-sis-b4;
SEQ ID NO:62 human c-sis-b5;
SEQ ID NO:63 human c-snoA;
SEQ ID NO:64 human c-snoN;
SEQ ID NO:65 human c-spi-1;
SEQ ID NO:66 human c-src-1;
SEQ ID NO:67 human c-src-2;
SEQ ID NO:68 human c-src-3;
SEQ ID NO:69 human c-src-4;
SEQ ID NO:70 human c-src-5;
SEQ ID NO:71 human c-src-6;
SEQ ID NO:72 human c-src-7;
SEQ ID NO:73 human c-src-8;
SEQ ID NO:74 human c-src-9;
SEQ ID NO:75 human c-src-10;
SEQ ID NO:76 human c-src-11;
SEQ ID NO:77 human c-syn;
SEQ ID NO:78 human c-trk;
SEQ ID NO:79 human c-vav;

SEQ ID NO:80 human c-mos-OA;
SEQ ID NO:81 human GP5-mos;
SEQ ID NO:82 human c-yes-1;
SEQ ID NO:83 human c-yes-2;
SEQ ID NO:84 human c-ski-1;
SEQ ID NO:85 human c-ski-2;
SEQ ID NO:86 human c-ski-3;
SEQ ID NO:87 human c-ski-4;
SEQ ID NO:88 human c-ski-5;
SEQ ID NO:89 human c-myb-1;
SEQ ID NO:90 human c-myb-2;
SEQ ID NO:91 human c-myb-3;
SEQ ID NO:92 human c-myb-4;
SEQ ID NO:93 human c-rel.

Moreover, according to the present invention the subject oligonucleotides need have only sufficient complementarity to detectably bind to either strand of a target nucleic acid sequence, e.g. SEQ ID NO:1-98.

Complementarity between nucleic acids is the degree to which the bases in one nucleic acid strand can hydrogen bond, or base pair, with the bases in a second nucleic acid strand. Hence, complementarity can sometimes be conveniently described by the percentage, i.e. proportion, of nucleotides which form base pairs between two strands or within a specific region or domain of two strands. For the present invention sufficient complementarity means that a sufficient number of base pairs exists between the subject oligonucleotides and a target nucleic acid to achieve detectable binding of the oligonucleotide.

Therefore a sufficient number, but not necessarily all, nucleotides in the present oligonucleotides can hydrogen bond to a target. The number of positions which are necessary to provide sufficient complementarity for binding of the subject oligonucleotides, can be detected by standard procedures including a melting temperature determination, standard Southern and Northern hybridization, light absorption detection, gel shift, DNA footprinting, alkylation interference and related procedures (as provided for example in Sambrook et al.). Moreover, according to the present invention oligonucleotide binding can be detected functionally, e.g. by observing a decrease in cellular or viral proliferation or by observing a decrease or increase in the synthesis of the DNA, RNA or protein encoded within or by a template nucleic acid.

Accordingly the degree of complementarity between an oligonucleotide of the present invention and a strand of a target nucleic acid need not be 100% so long as oligonucleotide binding can be detected. However, it is preferred that the present oligonucleotides have at least about 50% complementarity with their target nucleic acids. In an especially preferred embodiment sufficient complementarity is greater than 70% complementarity with the target.

Moreover, the degree of complementarity that provides detectable binding between the subject oligonucleotides and the target is dependent upon the conditions under which that binding occurs. It is well known that binding between nucleic acid strands depends on factors besides the degree of mismatch between two sequences. Such factors include the GC content of the

region, temperature, ionic strength, the presence of formamide and types of counter ions present. The effect that these conditions have upon binding is known to one skilled in the art. Furthermore, conditions are frequently determined by the circumstances of use. For example, when an oligonucleotide is made for use in vivo, no formamide will be present and the ionic strength, types of counter ions, and temperature correspond to physiological conditions. Binding conditions can be manipulated in vitro to optimize the utility of the present oligonucleotides. A thorough treatment of the qualitative and quantitative considerations involved in establishing binding conditions that allow one skilled in the art to design appropriate oligonucleotides for use under the desired conditions is provided by Beltz et al., 1983, Methods Enzymol. 100: 266-285 and by Sambrook et al.

Thus for the present invention, one of ordinary skill in the art can readily design a nucleotide sequence for the subject oligonucleotides which exhibits sufficient complementarity to detectably bind to the target nucleic acid of interest including nucleic acids having SEQ ID NO: 1-93. To confirm a nucleotide sequence, oligonucleotides may be subjected to DNA sequencing by any of the known procedures, including Maxam and Gilbert sequencing, Sanger sequencing, capillary electrophoresis sequencing, the wandering spot sequencing procedure or by using selective chemical degradation of oligonucleotides bound to Hybond paper. Sequences of oligonucleotides can also be analyzed by plasma desorption mass spectroscopy or by fast atom bombardment (McNeal, et al., 1982, J. Am.

Chem. Soc. 104: 976; Viari, et al., 1987, Biomed. Environ. Mass Spectrom. 14: 83; Grotjahn et al., 1982, Nuc. Acid Res. 10: 4671). Sequencing methods are also available for RNA oligonucleotides.

A further aspect of this invention provides pharmaceutical compositions containing the subject oligonucleotides with a pharmaceutically acceptable carrier. In particular, the present invention provides a pharmaceutical composition for regulating biosynthesis of a nucleic acid or protein comprising a biosynthesis regulating amount of the subject oligonucleotide with a pharmaceutically acceptable carrier.

As used herein a biosynthesis regulating amount of the subject oligonucleotides is about 0.1 µg to about 100 mg per kg of body weight per day, and preferably of about 0.1 µg to about 10 mg per kg of body weight per day. Dosages can be readily determined by one of ordinary skill in the art and formulated into the subject pharmaceutical compositions.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The subject oligonucleotides can be provided to a mammalian cell by topical or parenteral

administration, for example, by intraveneous, intramuscular, intraperitoneal subcutaneous or intradermal route, or when suitably protected, the subject oligonucleotides can be orally administered. The subject oligonucleotides may be incorporated into a cream, solution or suspension for topical administration. For oral administration, oligonucleotides may be protected by enclosure in a gelatin capsule. Oligonucleotides may be incorporated into liposomes or liposomes modified with polyethylene glycol for parenteral administration. Incorporation of additional substances into the liposome, for example, antibodies reactive against membrane proteins found on specific target cells, can help target the oligonucleotides to specific cell types.

Topical administration and parenteral administration in a liposomal carrier is preferred.

The following examples further illustrate the invention.

EXAMPLE 1

A METHOD FOR MAKING AN Rp STEREOISOMERIC ALKYLPHOPHONATE LINKAGE

Reactions for producing an Rp-stereospecific linkage are depicted below in Reaction Scheme I. DMT is used for dimethoxytrityl in Reaction Scheme I.

A 3'-O-methylphosphonoamidite nucleotide (1) is obtained by known procedures (e.g. Agrawal et al. 1987 Tetrahedron Letters 28: 3539-3542). In the first step, 1 mMole of 1 is hydrated with 5 mMole of tetrazole in 10 ml water for 1 min at room temperature, to produce a racemic methylphosphinate nucleotide (2Rp and 2Sp). The Rp and Sp stereoisomers of racemic 2 are stable and can be separated by chromatography on CH_3COOH/methanol washed silica with $CHCl_3$/methanol elution.

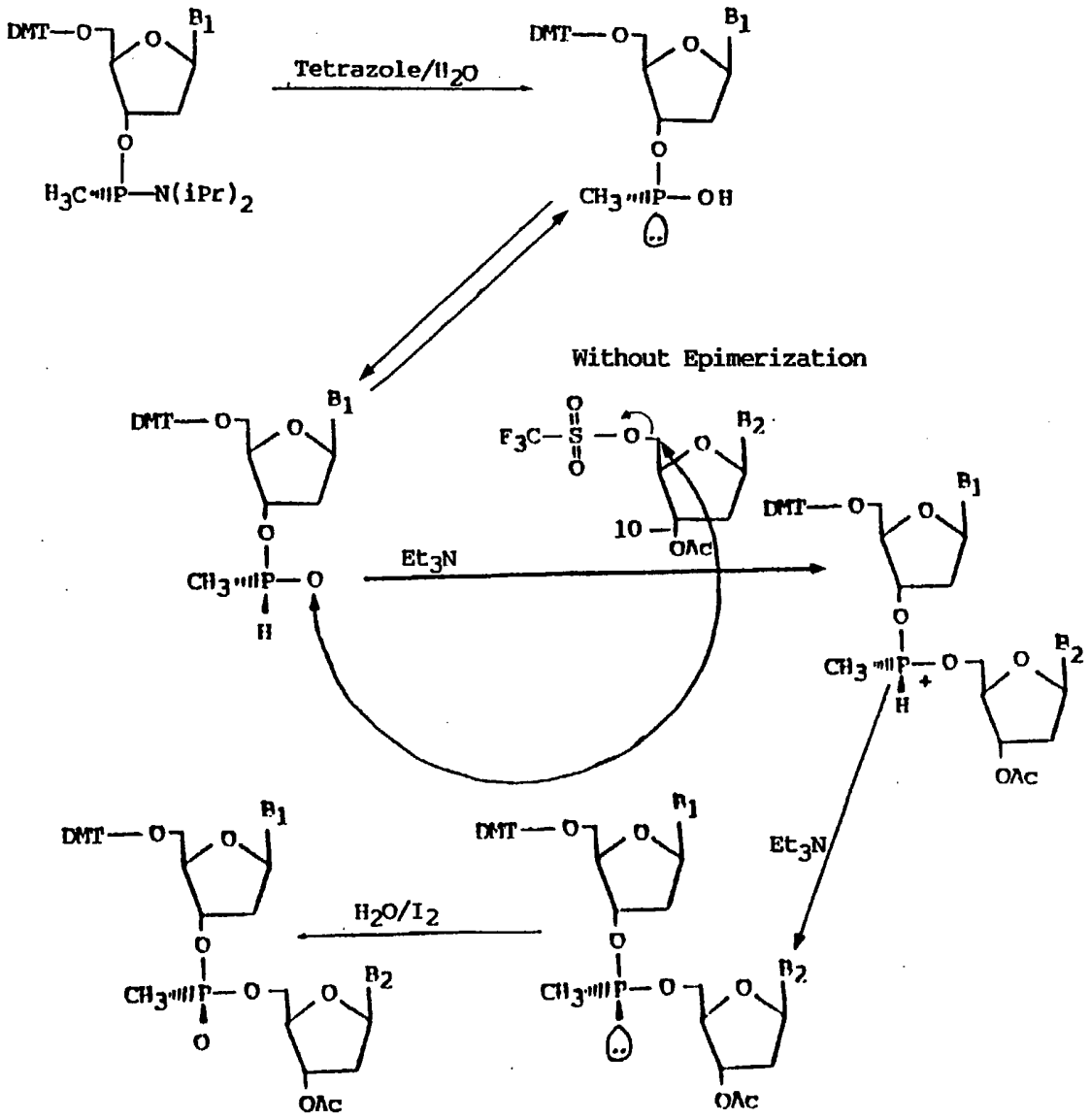
To produce an activated 5'-O-activated nucleotide triflate (3) which can be reacted with the Sp methylphosphinate intermediate (2Sp), the 5'-OH group of a nucleotide (4) was first replaced with an iodine. Subsequently, __ mMole 5'-iodo-3'-acetyl nucleoside (5) was reacted with __ mMole silver trifluoromethyl-sulfonate (6) for __ min at room temperature (Reaction Scheme III). The resulting 5'-O-activated nucleotide triflate (3) can be purified by silica gel HPLC using toluene-acetonitrile (3:2) as an eluent. Storage of such an activated 5'-O-activated triflate of thymidine in dimethylsulfoxide for several weeks did not lead to significant decomposition, as measured by ^{31}P nuclear magnetic resonance (NMR). Before coupling, the 5'-O-activated triflate (3) is dried by evaporation from anhydrous acetonitrile.

The 5'-O-activated triflate (3) is then coupled to the 5' position of the methylphosphinate intermdiate (2Sp) without altering the Sp phosphorus configuration. This coupling reaction is performed under anhydrous conditions by placing __ mMole 2Sp in 10 ml acetonitrile and 1 ml triethylamine and then adding 0.5 mMole of 3. The reaction is allowed to proceed at room temperature for 5 min and yields a dinucleotide (7) wherein the 5'-oxygen of the triflate (3) is displaced by an oxygen present on the phosphorus of the methylphosphinate (2Sp). The resulting Sp methylphosphonate dinucleotide (7) is then deprotonated to generate a trivalent methylphosphinate Sp linkage 8 by addition of 1 ml triethylamine to 7 in 10 ml acetonitrile and incubation for 1 min at room temperature. The Sp stereoisomer of 9 is stable since a distinct ^{31}P NMR peak corresponding to 9 was observed during NMR observation of the coupling reaction.

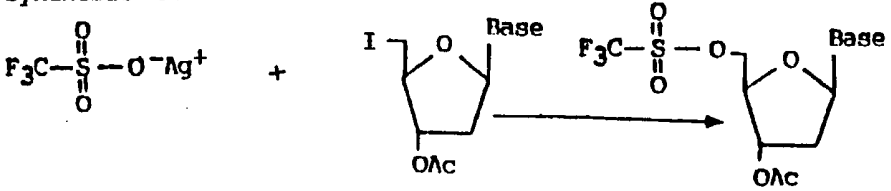
The Sp configuration of the deprotonated linkage 9 is inverted by oxidation using 1 mMole I_2 in 10 ml water for 5 min to produce the Rp stereoisomer of the methylphosphonate dinucleotide 10.

To regenerate the new 5'-terminal OH and thereby allow addition of new Rp phosphonate linkage, the 5'-DMT is removed and the resulting 5'-OH is activated by iodination followed by reaction with silver trifluoromethyl-sulfonate (6) to produce a new 5'-O-activated triflate (3).

REACTION SCHEME 1



Synthesis of the Triflate



EXAMPLE 2

METHODS FOR DETECTING AND MONITORING THE STEREOISOMERIC CONFIGURATION OF A PHOPHONATE LINKAGE

Separation of Stereoisomers:

Rp and Sp stereoisomers of alkyl- or aryl-phosphonate nucleotides prepared as in Example 1, were stable and were separated by ion exchange chromatography or by high pressure liquid chromatography (HPLC) using anhydrous or aqueous solvents. Reversed phase or silica gel columns were employed when separation was by HPLC. For example, Sp- and Rp-stereoisomers of 5'-dimethoxytritylthymidyl-3'-methylphosphinate were separated by HPLC using acetic acid/methanol washed C_{18} silica gel and $CHCl_3$/methanol as an eluent.

Similarly, racemic 5',3'-protected dithymidine methylphosphonate was resolved into Rp and Sp stereoisomers by HPLC on a 4.6 x 250 mm column of silica gel a gradient of 10-15% acetonitrile in water for elution (Fig. 1). Accordingly, Rp and Sp stereoisomers of both nucleotides and short oligonucleotides can be chromatographically separated.

Detection by Circular Dichroism:

Circular dichroism (CD) has been used to detect stereoisomeric differences. For example, separate Rp and Sp stereoisomers of dithymidine methylphosphonate have different CD spectra, wherein the Rp isomer has a larger CD peak and the Sp isomer CD trough is blue-shifted (Fig. 2).

Detection by Nuclear Magnetic Resonance:

Separated Rp and Sp stereoisomers have distinctive ^1H and ^{31}P nuclear magnetic resonance (NMR) spectra. For example, Figs. 3 and 4 depict the

respective ^{1}H and ^{31}P NMR spectra of both Rp and Sp stereoisomers of dithymidine methylphosphonate.

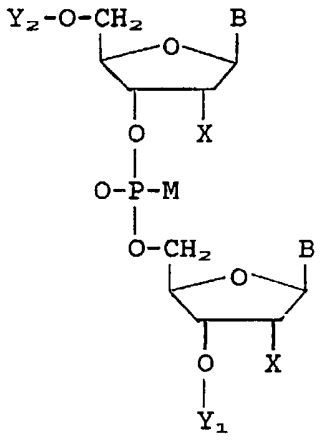
Detection by Mass Spectroscopy:

Fast atom bombardment mass spectrometry (FABMS) has been used extensively to examine the structures of oligonucleotides having molecular weights up to 10,000 g/mole (Stec et al. 1985 J. Org. Chem. 50: 3908; Ulrich et al. 1984 Org. Mass Spectrom. 19: 585; Grotjahn et al. 1982 Nucleic Acids Res. 10: 4671; Grotjahn et al. 1983 Int. J. Mass Spectrom. Ion Phys. 46: 439; Sindona et al. 1982 J. Chem. Res. (S):184; Eagles et al. 1984 Biomed. Mass. Spectrom. 11: 41; Connolly et al. 1984 Biochemistry 23: 3443-3453; and Matsuo et al. 1986 34th Annual Conference on Mass Spectrometry and Allied Topics, 329). Therefore, FABMS has utility for structural analyses of R and S stereoisomers of alkyl- and aryl-phosphonates.

For example, FABMS of tetrathymidine methylphosphonate (i.e. DMT-TpTpTpT-OAc) which was sputtered from thioglycerol yielded the spectrogram depicted in Fig. 5 wherein peaks corresponding to distinct molecular fragments are identified (e.g. DMT-TpT is dimethoxytrityl-dithymidine methylphosphonate).

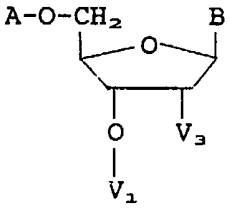
WHAT IS CLAIMED:

1. A method for producing an oligonucleotide having an Rp stereoisomeric alkyl- or aryl-phosphonate linkage between a first nucleotide and a second nucleotide in the oligonucleotide, wherein said oligonucleotide has the formula:

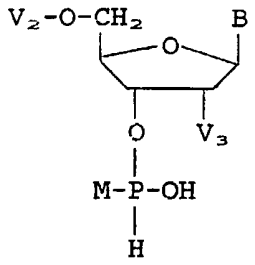


which comprises:

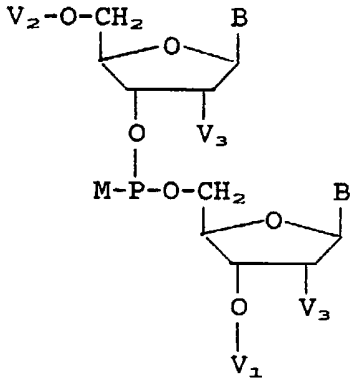
(a) reacting a 5'-O-activated nucleotide of the formula:



with an alkyl- or aryl-phosphinate nucleotide intermediate of the formula:



under conditions sufficient to produce an Sp stereoisomeric alkyl- or aryl-phosphonate linkage of the formula:



wherein:

Y_1 is a hydrogen, phosphate, phosphate present in said oligonucleotide or V_1;

Y_2 is a hydrogen, phosphate, phosphate present in said oligonucleotide or V_2;

X is hydroxy or V_3;

V_1 is a protecting group, solid support or phosphate present on the penultimate nucleotide of said oligonucleotide;

V_2 is a protecting group;

V_3 is hydrogen or OY_3 wherein Y_3 is lower alkyl or protecting group;

M is a lower alkyl, cycloalkyl, thioxo, a thio-lower alkyl, aryl or aryl-lower alkyl group which can be substituted with at least one hydroxy, halogen or cyano group;

each B group is independently a purine or pyrimidine base which can have 1-3 substituents selected from the group consisting of lower alkyl, amino, oxo, hydroxy, lower alkoxy, amino-lower alkyl, lower alkylamino, hydroxy-lower alkyl, aryl and aryl lower alkyl;

A is an activating group; and

said intermediate has an Sp phosphorus stereoisomeric configuration;

(b) reacting said Sp linkage with an oxidizing agent under conditions sufficient to produce said Rp stereoisomeric alkyl- or aryl-phosphonate linkage; and

(c) when V_1, V_2 or V_3 is a protecting group, optionally removing said V_1, V_2 or V_3 protecting group.

2. A method of producing at least one Rp-alkyl-phosphonate or Rp-aryl-phosphonate linkage in a polynucleotide chain of an oligonucleotide, wherein said oligonucleotide has the formula: